GENE THERAPY USING ANTI-gp41 ANTIBODY AND CD4 IMMUNOADHESIN

This invention discloses a method for treating infectious diseases, in particular viral HIV-1 infection, by gene therapy, and the genetic recombinant means to implement this treatment.

One means of combating HIV-1 infection would be to provide seropositive patients with passive immunoserotherapy, using soluble molecules directed against the virus, most particularly viral proteins, to obtain the neutralization of HIV *in vivo* [10]. Experiments involving the inoculation of virus into monkeys, together with the administration of anti-HIV-1 immunoglobulins, demonstrated the feasibility of the prevention of infection [11,13]. Histopathological, immunological and virological characteristics in the protected animals were strikingly similar to those observed in long-term human survivors with non-progressive HIV-1 infection [14].

An anti-gp41 monoclonal antibody (2F5mAb) [36] directed against the envelope glycoprotein gp41 of HIV-1 displayed a potent neutralization effect *in vitro* and *in vivo*, either alone or in various combinations with other monoclonal antibodies or with hyperimmune globulins [15,13,16,17].

The neutralization of HIV-1 mediated by a soluble form of CD4 (sCD4) which is the primary receptor for viral attachment to the envelope glycoprotein gp120 has also been demonstrated, either alone [18,19,49] or in combination with V3 loop monoclonal antibodies [20]. sCD4 acts as a decoy towards HIV-1 gp120 thus limiting the binding of this retroviral glycoprotein onto the CD4 receptor of T-cells, which is the key for entering and infecting the cells.

WO-94/19017 [49] discloses a pharmaceutical composition comprising sCD4, 2F5 monoclonal antibody and a

carrier, which is administered to an HIV-1-infected patient, in an amount effective to reduce the rate of spread of the HIV-1 and infection.

Moreover, in each of the above-mentioned prior art therapeutic solutions, the treatment induces in the patient a growing immunological response directed against the therapeutic molecule. Consequently, the necessary quantities of the serotherapeutic agent rapidly increase as the treatment goes on, up to quantities that are no longer compatible for human administration, since they may amount to huge volumes after several weeks of treatment. In this respect, passive immunoserotherapy suffered limitation as a treatment option, and has thus been abandoned.

It has been evidenced that delivering sCD4, by gene therapy, provided an efficient inhibition of HIV-1 infection *in vitro* [23]. Morgan RA et al. have actually reported that in a co-culture of either human T-cell lines or primary T-cells with sCD4-producing NIH 3T3 cells, the T-cell lines or T-cells obtained by the coculture were protected after HIV-1 challenge.

This therapy which may also apply to HIV-2 infection as the envelope glycoprotein gp105 of HIV-2 binds to CD4 receptor, is nevertheless insufficient and may not be safe, because, firstly, in case the gene encoding the said sCD4 is not expressed in the host cell, no therapeutic effect will affect the patient, and secondly, even if the binding site of the glycoprotein gp120 with said CD4 receptor is rather constant from one HIV-1 isolate to another, the least mutation in this region would result in a total failure of the treatment. It should be reminded that HIV-1 is naturally a highly variable retrovirus, and that the AIDS dual- and triple-therapy based on antiviral drugs has greatly promoted the generation of mutants.

There is still a great need for an efficient therapy that could apply to most HIV-1 strains.

According to the present invention, the anti-gp41 2F5mAb and a sCD4-based molecule were used for the first time in an efficient dual gene therapy.

The inventors have now discovered first that the production of both 2F5 monoclonal antibody and said sCD4-based molecule can be maintained continuously *in vivo*, and secondly that this production leads to a stable neutralization of HIV-1 by persistently and efficiently reducing the HIV-1 load *in vivo*.

The present invention provides a composition comprising one or more a nucleotide fragments, wherein the one or more nucleotide fragments taken together comprise at least (1) an nucleotide sequence (a) encoding human soluble CD4, (2) an nucleotide sequence (b) comprising at least nucleotide sequences encoding the heavy chain and the light chain of immunoglobulin IgG3, said IgG3 being directed against at least one of the peptide selected from the group consisting of SEQ ID NO:2 to SEQ ID NO:26, and (3) the nucleic elements required for replicating each said nucleotide sequences (a) and (b), in a host cell, when said host cell divides and for expressing under control each of said nucleotide sequences (a) and (b) in said host cell.

The composition according to the invention may comprise:

- an expression cassette comprising at least nucleotide sequence (a) and nucleotide sequence (b) and the nucleic elements required for expressing them under control in said host cell;

- a first expression cassette comprising nucleotide sequence (a), and the nucleic elements required for expressing it under control in said host cell, and a second expression cassette comprising nucleotide sequence

(b), and the nucleic elements required for expressing it under control in said host cell;

- a vector comprising at least nucleotide sequence (a) and nucleotide sequence (b), and the nucleic elements required for expressing them under control in said host cell;

- at least, a first recombinant vector comprising nucleotide sequence (a), and the nucleic elements required for expressing it under control in said host cell, and a second recombinant vector comprising nucleotide sequence (b), and the nucleic elements required for expressing it under control in said host cell.

In order to obtain constitutive secretion, that is a continuous expression, of 2F5mAb or of a monoclonal antibody directed against the same epitope as the one against which 2F5mAb is directed, said epitope consisting of an antigenic nucleotide sequence selected among the group consisting of SEQ ID NO:2 to SEQ ID NO:26, and of sCD4, *in vivo*, the inventors have first developed cell lines genetically modified by the vectors of the invention, and which have then been incorporated in collagen fibers or synthetic tissues to form neo-organs that could be grafted intraperitoneally in SCID mice.

Severe combined immune deficient (SCID) mice which are acknowledged animal models, have been used as recipients for human cell grafts [6,7]. Such humanized SCID mice were then shown to be readily infectable with HIV [8], and this model was recently used to test experimental gene therapy delivering interferons against HIV-1 infection [9].

As it will be illustrated in the examples, three types of neo-organs (2F5, sCD4-IgG, and 2F5 + sCD4-IgG) were generated to provide long-term delivery of recombinant molecules *in vivo*. These neo-organs became strongly vascularized within a few weeks of their implantation; they were not rejected and ensured secretion

of the desired molecules into the blood stream of the animals. This experimental model has been evidenced remarkable by the inventors for short term observations.

According to the invention, the neo-organs have been obtained from NIH3T3 murine fibroblasts genetically modified *in vitro*, by transduction with a recombinant vector of the invention. Their implantation into SCID mice led the continuous production of 2F5 which could be obtained for up to 6 weeks at serum concentrations close to 1 µg/ml.

Similarly, a soluble form of the CD4 molecule has been shown to block the interaction of CD4 cell with gp120, thus inhibiting HIV infection [37,38] as mentioned above. It was further demonstrated that the effect was dose-dependent when sCD4 cells were maintained throughout cell culture [39]. The continuous secretion of sCD4 by somatic transgenesis ensured their presence for 2 months after a single transgenesis in mice, whereas administration *in vivo* resulted in the short-term presence of the molecule, for a few hours [40]. Therefore, the inventors used a second-generation CD4-based molecule (named sCD4-IgG) which involves the genetic coupling of a portion of the CD4 structure (sCD4) to the Fc fragment of an IgG molecule. Such stabilized immunoglobulins or immunoadhesins [41] displayed the same affinity as sCD4 for gp120 and have a longer half-life *in vivo* [42].

In previous pilot experiments, the inventors confirmed the antiviral efficacy of the 2F5 recombinant molecules. Using 2F5 neo-organs, they observed that the intensity of the antiviral effect was dependent on the dose of antibody produced *in vivo*. It was found that plasma levels of 2F5 approximating 1 µg/ml in SCID mice were required to induce a regular and significant reduction in the virus load. In the present experiments, supporting the present invention, with the endogeneous production of 2F5 after neo-organ implantation, not only

was the number of RNA copies of HIV-1 in SCID-CEM mice very significantly decreased, but also the cellular viral load and reverse transcriptase activity were reduced.

Furthermore, when produced by neo-organs *in vivo*, the sCD4-IgG molecule is effective in significantly reducing the viral load of HV-infected SCID-CEM mice.

Either 2F5 or sCD4-IgG can inhibit HIV infection *in vivo*. Their potential antiviral efficiency was directly related to their ability to interact with the HIV-1 envelope. This would affect HIV-1 propagation by preventing the virus from infecting its target cells because of their competitive or neutralizing properties.

It has been discovered that when both 2F5 and sCD4-IgG are produced together, concomitantly or more or less concomitantly, a cooperation or complementary effect between 2F5 and sCD4 occurs in vivo. This effect which is evidenced in examples provides a safety degree of the treatment much higher than the sCD4 single agent therapy, above-mentioned, in that the treatment is effective irrespectively of the mutations or variability of the infecting virus, and reproducible from one mammal or patient to another one.

It is believed that the binding of sCD4-IgG on gp120 would facilitate the binding of 2F5 on gp41, by inducing a conformational change of the envelope and therefore increases the action of neutralizing 2F5 antibodies.

This complementary effect could result from the following mechanism: gp120 is a large and readily accessible glycoprotein which hinders the access to gp41; gp41 is a smaller glycoprotein and is "fitted" onto gp120. The binding of sCD4-IgG to the gp120 modifies the conformation of gp120, freeing the epitope region of gp41. As this access is made easier, 2F5 antibodies readily and efficiently bind to gp41, enhancing consequently the neutralizing activity of the combination sCD4-IgG and 2F5

compared to the simple additive effect of sCD4-IgG and 2F5.

Definition:

Human immunodeficiency virus type 1 (HIV-1) is the principal etiologic agent of AIDS as described by Barré-Sinoussi F et al. [51], Gallo RC et al. [52], and which nucleotide sequence has been reported by Wain-Hobson S et al. [53] and Ratner L et al. [54].

Soluble CD4 (sCD4) is an extracellular part of the human CD4 glycoprotein receptor of T-cells interacting with HIV-1 [55], said extracellular part of CD4 consisting of four variable domains, D1, D2, D3 and D4, bound by joining domains, J1, J2, J3 and J4, respectively and of a leader sequence L, and may represented for example by SEQ ID NO:32. According to the present invention, sCD4 is a polypeptide comprising at least L, D1, J1 and D2 domains. Preferably, sCD4 is a recombinant peptide illustrated by SEQ ID NO:33, encoded by SEQ ID NO:31. sCD4 is soluble in an aqueous solution including detergent-free aqueous buffer and body fluids such as blood, plasma and serum.

sCD4 multimer is a recombinant multimer protein comprising at least four segments each consisting of L, D1, J1 and D2 domains of CD4 (refer to WO-97/04109).

sCD4-IgG is a fusion protein which results in the fusion of sCD4 and the constant region of an immunoglobulin, for example 2F5mAb which preparation is described in WO-96/08574.

2F5 [36] is a human monoclonal antibody directed to the epitope sequence SEQ ID NO:2 belonging to the external domain of the gp41 envelope glycoprotein of most HIV-1 strains, or to an immunological equivalent sequence selected from the group consisting of SEQ ID NO:3-SEQ ID NO:26 [56,57]. 2F5mAb may be purchased from Virus Testing Systems (Houston, USA) and hybridoma cell line producing said mAb may be prepared from peripheral blood

mononuclear cells from HIV-1-infected patients which are then fused, and selected as described by Buchacher M. et al. [36].

The nucleotide sequences encoding respectively the light and the heavy chains of 2F5 may be obtained as follows:

The complementary DNA (cDNA) encoding the light chain is included in *Hind*III-*Eco*RI fragment in vector Bluescript SK+ (purchased from Stratagène). This cDNA has been obtained from a cDNA library originating from the mRNA of hybridoma 2F5 [28] by using primers identified by SEQ ID NO:27 and 28.

The complementary DNA (cDNA) encoding the heavy chain is included in *Nco*I-*Eco*RI fragment in vector pTG2677 described in Figure 1. This cDNA has been obtained from a cDNA library originating from the mRNA of hybridoma 2F5 [28] by using primers identified by SEQ ID NO:29 and 30.

Nucleic elements contained in a composition, in an expression cassette or in a vector of the invention, and which is required for expressing specified sequences, under control, in a host cell, may be exogenous or endogenous elements; this expression is carried out under control, that is the control of at least one step of the expression process, selected among the group consisting of transcription, maturation of RNA, transport of RNA, translation, degradation.

A neo-organ, also designated as organoid or implant, is an organ which has been obtained in laboratory, starting from human or animal cells that are cultivated *in vitro* on an extra-cellular polymer matrix. When the cells have reached the desired growth level, the neo-organ is implanted in a human or animal. According to the invention, said implant comprises living modified cells which are able to produce at least one molecule of interest. The neo-organ thus implanted is capable of continuously releasing in the recipient, *in vivo*, said

molecule of interest. The matrix is preferably made of at least one bio-compatible material selected from collagen, polytetrafluoroethylene, Gore-Tex™ [75]

The nucleotide sequences (a) and (b) to which the present invention pertains can be cDNA or genomic sequences or be of a mixed type. It can, where appropriate, contain one or more introns, with these being of native, heterologous (for example the intron of the rabbit β-globin gene, etc.) or synthetic origin, in order to increase expression in the host cells.

The nucleotide sequences employed within the context of the present invention can be obtained by the conventional techniques of molecular biology, for example by screening libraries with specific probes, by immunoscreening expression libraries or by PCR using suitable primers, or by chemical synthesis.

As previously mentioned, the present invention also relates to at least one recombinant vector, or two recombinant vectors which comprise nucleotide sequences (a) and (b) according to the invention, which is placed under the control of the nucleic elements which are required for expressing it in a host cell.

A vector according to the present invention, or nucleic acid construct, devoted to gene therapy, may be used in its naked form [58], combined with liposomes, cationic lipids, cationic polymers, peptides or polypeptides. The literature relating to the vectors that may be used in gene therapy provides a considerable numbers of examples of such vectors [see for example 59].

The recombinant vectors can be of plasmid or viral origin and can, where appropriate, be combined with one or more substances which improve the transfectional efficiency and/or stability of the vectors. These substances are widely documented in the literature which is available to the skilled person (see, for example, 60, 61, 62]. By way of non-limiting illustration, the

substances can be polymers, lipids, in particular cationic lipids, liposomes, nuclear proteins or neutral lipids. These substances can be used alone or in combination. A combination which can be envisaged is that of a recombinant plasmid vector which is combined with cationic lipids (DOGS, DC-CHOL, spermine-chol, spermidine-chol, etc.) and neutral lipids (DOPE).

The choice of the plasmids which can be used within the context of the present invention is immense. They can be cloning vectors and/or expression vectors. In a general manner, they are known to the skilled person and, while a number of them are available commercially, it is also possible to construct them or to modify them using the techniques of genetic manipulation. Examples which may be mentioned are the plasmids which are derived from pBR322 (Gibco BRL), pUC (Gibco BRL), pBluescript (Stratagene), pREP4, pCEP4 (Invitrogene) or p Poly [63]. Preferably, a plasmid which is used in the context of the present invention contains an origin of replication which ensures that replication is initiated in a producer cell and/or a host cell (for example, the oriP/EBNA1 system will be chosen if it's desired that the plasmid should be self-replicating in a mammalian host cell [64,65]). The plasmid can additionally comprise a selection gene which enables the transfected cells to be selected or identified (complementation of an auxotrophic mutation, gene encoding resistance to an antibiotic, etc.). Certainly, the plasmid can contain additional elements which improve its maintenance and/or its stability in a given cell (*cer* sequence) which promotes maintenance of a plasmid in monomeric form [66].

The recombinant vectors may be independently selected from adenoviral vectors, lentiviral vectors [67], "new generation" adenoviral vectors, retroviral vectors, vectors which are derived from a poxvirus (vaccinia virus, in particular MVA, canarypoxvirus, etc.), vectors which

are derived from a herpesvirus, from an alphavirus, from a foamy virus or from an adenovirus-associated virus, chimeric viral vectors and synthetic vectors [68].

Retroviruses have the property of infecting, and in most cases integrating into, dividing cells and in this regard are particularly appropriate for use in relation to cancer. A recombinant retroviral vector according to the invention generally contains the LTR sequences, an encapsidation region and at least one of the nucleotide sequence according to the invention, which is placed under the control of the retroviral LTR or of an internal promoter such as those described below. The recombinant retroviral vector can be derived from a retrovirus of any origin (murine, primate, feline, human, etc.) and in particular from the MoMuLV (Moloney murine leukemia virus), MVS (Murine sarcoma virus) or Friend murine retrovirus (Fb29). It is propagated in an encapsidation cell line which is able to supply *in trans* the viral polypeptides gag, pol and/or env which are required for constituting a viral particle. Such cell lines are described in the literature (PA317, Psi CRIP GP + Am-12 etc.). The retroviral vector according to the invention can contain modifications, in particular in the LTRs (replacement of the promoter region with a eukaryotic promoter) or the encapsidation region (replacement with a heterologous encapsidation region, for example the VL30 type).

Preference will be given to using a vector which does not replicate and does not integrate. In this respect, adenoviral vectors are very particularly suitable for implementing the present invention.

Accordingly, the prefered use is of an adenoviral vector which lacks all or part of at least one region which is essential for replication and which is selected from the E1, E2, E4 and L1-L5 regions in order to avoid the vector being propagated within the host organism or

the environment. A deletion of the E1 region is preferred. However, it can be combined with (an)other modification(s)/deletion(s) affecting, in particular, all or part of the E2, E4 and/or L1-L5 regions, to the extent that the defective essential functions are complemented in *trans* by means of a complementing cell line and/or a helper virus. In this respect, it is possible to use "new generation" vectors of the state of the art. By way of illustration, deletion of the major part of the E1 region and of the E4 transcription unit is very particularly advantageous. For the purpose of increasing the cloning capacities, the adenoviral vector can additionally lack all or part of the non-essential E3 region. According to another alternative, it is possible to make use of a minimal adenoviral vector which retains the sequences which are essential for encapsidation, namely the 5' and 3' ITRs (Inverted Terminal Repeat), and the encapsidation region. The various adenoviral vectors, and the techniques for preparing them, are known [see, for example 69].

Furthermore, the origin of the adenoviral vector according to the invention can vary both from the point of view of the species and from the point of view of the serotype. The vector can be derived from the genome of an adenovirus of human or animal (canine, avian, bovine, murine, ovine, porcine, simian, etc.) origin or from a hybrid which comprises adenoviral genome fragments of at least two different origins. More particular mention may be made of the CAV-1 or CAV-2 adenoviruses of canine origin, of the DAV adenovirus of avian origin or of the Bad type 3 adenovirus of bovine origin [70,71,72,73]. However, preference will be given to an adenoviral vector of human origin which is preferably derived from a serotype C adenovirus, in particular a type 2 or 5 serotype C adenovirus.

An adenoviral vector according to the present invention can be generated *in vitro* in *Escherichia coli*

(E. coli) by ligation or homologous recombination or else by recombination in a complementing cell line.

The nucleic elements required for expression consist of all the elements which enable the nucleotide sequence to be transcribed into RNA and the mRNA to be translated into polypeptide. These elements comprise, in particular, a promoter which may be regulatable or constitutive. Logically, the promoter is suited to the chosen vector and the host cell. Examples which may be mentioned are the eukaryotic promoters of the PGK (phosphoglycerate kinase), MT (metallothionein) [84], α-1 antitrypsin, CFTR, surfactant, immunoglobulin, β-actin [76] and SRα [77] genes, the early promoter of the SV40 virus (Simian virus), the LTR of RSV (Rous sarcoma virus), the HSV-1 TK promoter, the early promoter of the CMV virus (Cytomegalovirus), the p7.5K pH5R, pK1L, p28 and p11 promoters of the vaccinia virus, and the E1A and MLP adenoviral promoters. The promoter can also be a promoter which stimulates expression in a tumor or cancer cell. Particular mention may be made of the promoters of the MUC-1 gene, which is overexpressed in breast and prostate cancers [78], of the CEA (standing for carcinoma embryonic antigen) gene, which is overexpressed in colon cancers [79] of the tyrosinase gene, which is overexpressed in melanomas [80], of the ERBB-2 gene, which is overexpressed in breast and pancreatic cancers [81] and of the α-fetoprotein gene, which is overexpressed in liver cancers [82]. The cytomegalovirus (CMV) early promoter is very particularly preferred.

The necessary elements can furthermore include additional elements which improve the expression of the nucleotide sequences (a) and/or (b) according to the invention or its maintenance in the host cell. Intron sequences, secretion signal sequences, nuclear localization sequences, internal sites for the reinitiation of translation of IRES type, transcription

termination poly A sequences, tripartite leaders and origins of replication may in particular be mentioned. These elements are known to the skilled person.

The invention also relates to:

- a host cell which comprises at least an expression cassette of the invention as previously described;

- a host cell which comprises at least a vector of the invention as previously described;

- wherein said host cell may be selected among the group consisting of fibroblasts, lymphocytes and stem cells;

- a tissue of genetically modified cells comprising a plurality of host cells previously described.

- an implant of genetically modified cells comprising a plurality of host cells previously described.

- said tissue or implant wherein said host cells are selected among the group consisting of fibroblasts, lymphocytes, stem cells.

The invention also relates to a method for treating an infectious disease, wherein the following medication may be administered by gene therapy, to a mammal or a patient, in particular when said infectious disease is caused by HIV-1 retrovirus:

- a composition of the invention as previously described

- at least (1) a first expression cassette comprising a nucleotide sequence (a) encoding human soluble CD4 and nucleic elements required for replicating nucleotide sequence (a) in a host cell, when said host cell divides, and for expressing under control said nucleotide sequence (a) in said host cell, (2) a second expression cassette comprising a nucleotide sequence (b) comprising at least nucleotide sequences encoding the heavy chain and the light chain of immunoglobulin IgG3, said IgG3 being directed against at least one of the

peptide selected from the group consisting of SEQ ID NO:2 to SEQ ID NO:26, and nucleic elements required for replicating nucleotide sequence (b), when said host cell divides, and for expressing under control said nucleotide sequence (b) in said host cell; said first expression cassette and said second expression cassette may be administered, concomitantly or separately;

- at least (1) a first recombinant vector comprising a nucleotide sequence (a) encoding human soluble CD4 and nucleic elements required for replicating nucleotide sequence (a) in a host cell, when said host cell divides, and for expressing under control said nucleotide sequence (a) in said host cell, (2) a second recombinant vector comprising a nucleotide sequence (b) comprising at least nucleotide sequences encoding the heavy chain and the light chain of immunoglobulin IgG3, said IgG3 being directed against at least one of the peptide selected from the group consisting of SEQ ID NO:2 to SEQ ID NO:26, and nucleic elements required for replicating nucleotide sequence (b), when said host cell divides, and for expressing under control said nucleotide sequence (b) in said host cell; said first recombinant vector and said second recombinant vector may be administered, concomitantly or separately;

- at a least one host cell of the invention as previously described;

- an implant of the invention as previously described.

A composition according to the invention can be made conventionally with a view to administering it locally, parenterally or by the digestive route. In particular, a therapeutically effective quantity of the therapeutic or prophylactic agent is combined with a pharmaceutically acceptable excipient. It is possible to envisage a large number of routes of administration. Examples which may be mentioned are the intramuscular,

intratracheal, intratumoral, intragastric, intraperitoneal, epidermal, intracardiac, intraperitoneal, intravenous or intraarterial route, by inhalation, by instillation, by aerosolization, by the topical route or by the oral route. In the case of these three latter embodiments, it is advantageous for administration to take place by means of an aerosol or by means of instillation. The administration can take place as a single dose or as a dose which is repeated on one or more occasions after a particular time interval. The appropriate route of administration and dosage vary depending on a variety of parameters, for example the individual to be treated, the vector which has been selected for the gene therapy. For example, the composition according to the invention can be formulated in the form of doses of about 890 ng/ml for 2F and 77 ng/ml for sCD4-IgG if the vector is of retroviral type, and in the form of doses of about 1 mg/ml for each of 2F5 and sCD4-IgG is the vector is of adenoviral type. Naturally, the doses can be adjusted by the clinician.

The composition can also include a diluent, an adjuvant or an excipient which is acceptable from the pharmaceutical point of view, as well as solubilizing, stabilizing and preserving agents. In the case of an injectable administration, preference is given to a formulation in an aqueous, non-aqueous or isotonic solution. It can be presented as a single dose or as a multidose, in liquid or dry (powder, lyophilizate, etc.) form which can be reconstituted at the time of use using an appropriate diluent.

The composition of the invention can be administered directly *in vivo* (for example by intravenous injection, into the lungs by means of an aerosol, into the vascular system using an appropriate catheter, etc.). It is also possible to adopt the *ex vivo* approach, which consists in removing cells from the patient (stem cells, peripheral blood lymphocytes, etc.), transfecting or

infecting them *in vitro* in accordance with the techniques of the art and then readministering them to the patient.

Figures:

Figure 1 is a schematic representation of the retroviral vector RVTG6371; RVTG6371 encodes the human monoclonal antibody (mAb) 2F5.

It carries the long terminal repeat of Moloney murine sarcoma virus (5'LTR), the Moloney murine sarcoma virus / Moloney murine leukemia virus hybrid packaging sequences (psi), the mouse phosphoglycerate kinase-1 gene promoter sequences (PGK), the mAb 2F5 heavy and light chain cDNA, (respectively 2F5 HC and 2F5 LC), the internal ribosome entry site (IRES) of encephalomyocarditis virus, the long terminal repeat (3'LTR) of myeloproliferative sarcoma virus, the puromycine acetyltransferase (Pac) gene and the simian virus polyadenylation (PA) signal.

Figure 2 is a schematic representation of the retroviral vector RVTG8338; RVTG8338 encodes the human soluble CD4-IgG immunoadhesin (sCD4-IgG).

Its construction is based on the ligation of the leader variable (V1/V2) segment of human CD4 (SEQ ID NO:30), 2F5 hinge region (Hinge CH_2-CH_3) and the 2F5 heavy chain (2F5 HC) sequence.

Figure 3 illustrates the *in vivo* secretion of 2F5 monoclonal antibody:NIH3T3TG6371 cells were implanted intraperitoneally into SCID mice; the presence of the 2F5 mAb, in animal sera was detected and followed for 2 months using enzyme-linked immunosorbent assay.

Figure 4 is a schematic representation of the structure of CD4 receptor.

Figure 5 is a schematic representation of a vector encoding the multimer protein sCD4.

Figure 6 represents the viral load in SCID-hu-HIV-1-sCD4-IgG-2F5 mice x1000(mRNA copies/ml) versus time in days.

Examples:

Examples of the present invention are also described in Sanhadji K. et al. [83], which is herein incorporated in its entirety by reference.

Example 1: Construction of vectors

The complementary DNA encoding the heavy chain (HC) and light chain (LC) of 2F5mAb have been obtained as indicated above, in the definition part of the description.

The sequence of sCD4-IgG results from the ligation of the segment L-D1-J1-D2 (SEQ ID NO:31) of human CD4 with the constant region of 2F5.

Both were inserted into retroviral vectors from Moloney murine leukemia virus, noted respectively RVTG6371 (illustrated on Figure 1) and RVTG8338 (illustrated on Figure 2). To avoid the gradual inactivation of retrovirally transferred expression cassette *in vivo*, the promoter of mouse phosphoglycerate kinase type I was used. High titres of transgene products were obtained with these constructions and extinction has never been observed *in vitro*.

The dicistronic vector RVTG6371, was used to give a sub-equivalent quantity of heavy chain sequence and light chain sequence of 2F5mAb directed against the ELDKWAS linear epitope (SEQ ID NO:2) of HIV-1 gp41.

The amount of sCD4-IgG obtained from RVTG8338 could not be quantified, but the molecules were detected by immunofluorescence, enzyme-linked immunosorbent assay (ELISA) and Western blot techniques.

Example 2: Construction of modified cell lines

NIH-3T3 fibroblasts were stably transduced with RVTG6371 or RVTG8338 encoding respectively 2F5mAb and sCD4-IgG, to obtain respectively NIH-3T3TG6371 and NIH-3T3TG8338. The resulting cells were shown constitutively to produce the 2F5mAb or the sCD4-IgG immunoadhesin

through the analysis of culture supernatants by a Western blot assay and ELISA and by a specific immunofluorescence technique, mentioned in Example 5B.

Example 3: Neo-organ construction

Neo-organs were built with the genetically modified fibroblasts obtained in Example 2, and a biocompatible material made of paratetrafluoroethylene (or Gore-Tex™) fibers coated with types III and I collagen threads and basic fibroblast growth factor (bFGF). The lattice of this artificial structure retracted within a few days in culture medium and the neo-organs were then ready for implantation into the peritoneal cavity of humanized severe combined immunodeficient (SCID) mice.

Example 4: Implantation of neo-organ in immunodeficient mice

Three groups of experiments were carried out: 2F5 (Group 2F5), sCD4-IgG (Group sCD4-IgG) or 2F5 plus sCD4-IgG (Group 2F5+sCD4-IgG) neo-organs were built and implanted into SCID mice as described below.

Ten SCID mice, controlled for agammaglobulinaemia were anaesthetized with phenobarbital. After median laparatomy, the neo-organs obtained in Example 3, maintained in bFGF-suplemented medium, were aseptically placed into the peritoneal cavity. These structures became strongly vascularized 1 or 2 weeks after their implantation in mice, as a result of the trophic and angiogenic properties of bFGF. SCID mice with neo-organs were bled weekly and checked for the presence of 2F5mAb or sCD4-IgG in serum samples. Three weeks after the neo-organ implantation, an optimal amount of recombinant molecules was reached.

At this time, 4×10^7 of human CD4 cells (CEM T cell line) were injected intraperitoneally. Animals receiving

CEM cells in the absence of previously implanted neo-organs were used as controls.

Two months after the implantation, SCID mice were killed to observe the structure and vascularization of the neo-organ.

Example 5: Detection of 2F5mAb and sCD4-IgG, *in vivo* and *ex vivo*

A) Enzyme-linked immunosorbent assay

Measures of 2F5 or sCD4-IgG *in vitro* and *ex vivo* were performed in cell culture supernatants and in plasma, respectively, using ELISA assays.

Briefly, microplates were coated by overnight incubation with purified ELDKWAS peptide (SEQ ID NO:2) or with gp160 proteins of the HIV-1 envelope. Inactivated plasma from neo-organ-implanted SCID mice from each group of Example 4, were then tested. 2F5mAb or sCD4-IgG were detected using horseradish peroxydase-conjugated goat anti-human IgG. The colored reaction was revealed using ortho-phenylenediamine as the substrate and stopped 3 minutes later by the addition of sulphuric acid 6 M. Optical densities were determined at 490 nm with the ELISA reader.

As evidenced on Figure 3, in neo-organ implanted SCID mice, the follow-up of 2F5 production showed an increased secretion during the first 5 weeks after grafting. The mean plasma levels of 2F5 increased from 167 to 1000 ng/ml between weeks 1 and 5 in Lai-inoculated group (represented by □), and from 114 to 2000 ng/ml between the same weeks in MN-inoculated group (represented by ■). At week 6, means of 450 and 1325 ng/ml 2F5 were found in the plasma of mice of the Lai and MN groups, respectively.

The inventors did not have any purified sCD4-IgG standard to quantify the production of the immunoadhesin *in vitro* and *in vivo*. Nevertheless, they were able to

detect the molecules in cell supernatants and in mouse plasma by a qualitative ELISA assay as described above.

B) Immunofluorescence detection of 2F5 mAb

Adherent cells were fixed for 20 minutes in methanol acetone (v/v) before being treated. One step of incubation with a fluorescein-isothiocyanate-conjugated goat antibody directed against human IgG (heavy and light chains) diluted at 1:200 in phosphate-buffered saline X 1 with 5% fetal calf serum was performed. Ethidium bromide 1:100 in phosphate-buffered saline X 1 was used to stain the nuclei.

Example 6: Detection of 2F5mAb and sCD4-IgG, *in vivo*

Measures of 2F5 and sCD4-IgG *in vivo* in [2F5+sCD4-IgG]-neo-organ-implanted SCID mice of Example 4 were performed using ELISA assays. These measures are carried out by a serum weekly assay.

A) Production of 2F5, *in vivo*

As already evidenced in Example 5A and as shown in Table 1 below, the 2F5 concentration in plasma rapidly increases as from the first week after the neo-organ implantation. The mean plasma levels of 2F5 increased from 60 ng/ml to 1450 ng/ml between the first and the third weeks.

B) Production of sCD4-IgG *in vivo*

First, a qualitative evaluation of sCD4-IgG secretion was performed using ELISA assays. The mean plasma levels of sCD4-IgG increased from 10 ng/ml to 77 ng/ml between the first and the third weeks after the neo-organ implantation.

Second, a quantitative evaluation of sCD4-IgG secretion was performed using ELISA assays. As shown in Table 1 below, the mean plasma levels of sCD4-IgG

increased from 4 ng/ml to 170 ng/ml between the first and the third weeks after the neo-organ implantation.

Table 1 also evidences that on the implantation day, neither 2F5, nor sCD4-IgG is secreted, *in vivo*.

Table 1

	Secretion of transgenic molecules in plasma (ng/ml)			
Mice	Week 0	Week 1	Week 2	Week 3
2F5				
SCID 1	0	120	360	710
SCID 2	0	240	420	1075
SCID 3	0	170	275	580
SCID 4	0	60	160	615
SCID 5	0	240	275	1450
SCID 6	0	100	170	460
SCID 7	0	160	225	1350
SCD4-IgG				
SCID 1	0	20	11	74
SCID 2	0	4	16	50
SCID 3	0	5	4	170
SCID 4	0	19	10	34
SCID 5	0	7	26	127
SCID 6	0	12	21	46
SCID 7	0	8	12	40

Example 7: HIV-1 infection *in vivo*

A) Mice of Example 4

HIV-1 challenge *in vivo*

Four weeks after the intraperitoneal injection of CEM cells, SCID mice of Example 4 were challenged intravenously with 1000 $TCID_{50}$ (50% tissue-culture infectious dose) of HIV-1 (Lai or MN). The virus titration was carried out in CEM cell cultures, using the Nara technique (Nara et al.).

Measures of plasma and cellular viral loads, and reverse transcriptase activity

On one part of the cells removed from the spleen, the cellular proviral DNA was analyzed, using a quantitative-competitive polymerase chain reaction (PCR). The total DNA was extracted according to the trireagent method (Euromedex, Strasbourg, France) from HIV-1-infected and from non-infected CEM cells harvested from the spleens of killed SCID mice.

The plasma viral load was measured using the NASBA kit. A 140 base pair fragment of the *gag* gene was amplified from 2 µg DNA by quantitative-competitive PCR (HIV-1 PCR MIMIC Quantitation System kit; Clontech, Cambridge Bioscience, Cambridge, UK) using a SK 462/SK 431 primer pair in the presence of a 260 base pair heterologous fragment. A 10-fold dilution of the competitor (10^6:1 copies) allowed the determination of the equimolariry of *gag* in each sample. The analysis of PCR products was performed by Southern blot hybridization using ^{32}P-labelled SK 102 and MIMIC probes (Clontech).

On the other part of the cells removed from the spleen, liver and tumor, cultures were performed to measure reverse transcriptase activity [Touraine JL, Sanhadji K. and Sembeil R. IMAJ, Gene therapy for HIV infection in the humanized SCID mouse model, in press].

B) Mice of Example 6

HIV-1 challenge *in vivo*

Three weeks after the intraperitoneal injection of CEM cells, SCID mice of Example 6 were challenged intravenously with 1000 $TCID_{50}$ (50% tissue-culture infectious dose) of HIV-1 (Lai). The plasma viral load is followed up by measure on day 0, 4 and 10 after HIV-1 inoculation. As illustrated on Figure 6, the plasmatic viral load rapidly increases as from four days after the infection (up to 8 logarithms)

Example 8: *In vivo* decrease of viral load induced by 2F5 mAb

A) *In vivo* decrease of plasma viral load induced by 2F5mAb

As shown in Table 2, seven control SCID mice (four Lai and three MN) of Example 4 and six SCID mice with 2F5 mAb-producing neo-organs (four Lai and two MN) of Group 2F5 of Example 4 were tested for plasma viral load.

Table 2

Mice	HIV-1-RNA copies/ml at :			
	Day 0	Day 5	Day 8	Day 12
Controls				
SCID 01 + Lai	$<16 \times 10^3$	$<16 \times 10^3$	75×10^7	$2,9 \times 10^8$
SCID 02 + Lai	$<16 \times 10^3$	$<16 \times 10^3$	66×10^7	12×10^8
SCID 03 + Lai	ND	$<8 \times 10^3$	64×10^7	9×10^8
SCID 04 + Lai	ND	$<13 \times 10^3$	94×10^7	12×10^8
SCID 05 + Lai	ND	ND	ND	ND
SCID 5′ + MN	ND	$<7 \times 10^3$	31×10	13×10^8
SCID 6′ + MN	ND	$<11 \times 10^3$	$9,5 \times 10^8$	$8,2 \times 10^8$
SCID 7′ + MN	ND	$<3 \times 10^3$	12×10^8	12×10^8
2F5 neo-organs				
SCID 8 + Lai	ND	$<27 \times 10^3$	ND	$<2,5 \times 10^3$
SCID 9 + Lai	ND	$<27 \times 10^3$	ND	$<3 \times 10^3$
SCID 10 + Lai	ND	$<18 \times 10^3$	ND	$<5,5 \times 10^3$
SCID 11 + Lai	ND	$<13 \times 10^3$	ND	$<6 \times 10^3$
SCID 12 + Lai	ND	ND	ND	ND
SCID 13 + Lai	ND	ND	ND	ND
SCID 14 + MN	ND	$<25,5 \times 10^3$	ND	$<16,5 \times 10^3$
SCID 15 = MN	ND	$<4 \times 10^3$	ND	$<4 \times 10^3$

ND: Not done

Statistical analysis:

For Lai group: 2F5 neo-organs versus controls $P < 0.0001$

For MN group: 2F5 neo-organs versus controls $P < 0.0002$

In control animals, the plasma viral load increased significantly from day 5 to day 12 after HIV-1 challenge. The number of RNA copies reached $2.9\text{-}12 \times 10^8$ and $8.2\text{-}13 \times 10^8$ for Lai and MN isolates, respectively.

In mice implanted with 2F5-producing neo-organs, the plasma viral load was approximately 100 000-fold lower than that in control mice at day 12, both in the Lai and the MN groups. All tested implanted mice had a number of RNA copies below 16.5×10^3 at day 12.

B) *In vivo* decrease of cellular viral load induced by 2F5mAb

At the time of death, quantitative detection of HIV-1 proviruses was performed by PCR in one part of the spleen cells recovered from control and from 2F5 neo-organ-implanted SCID mice inoculated with HIV-1. Proviral DNA was detected in all samples, but at different levels. It was approximately 100-fold lower in cells from mice with a 2F5 neo-organ than in cells from control mice. Whereas HIV-1 (Lai) experimental inoculation resulted in infection with either 3×10^6 or 3×10^5 HIV-1 copies in the five control mice, in SCID mice grafted with a 2F5 neo-organ, 3×10^5 HIV-1 copies, 3×10^4 HIV-1 copies or 3×10^3 HIV-1 copies were detected. In mice injected with the MN strain, no statistically significant difference in the detection of intracellular HIV-1 provirus was observed between controls and 2F5-producer animals.

Cell cultures were performed using human T cells (CEM cells) recovered from the second part of various organs (spleen, tumor and liver) of grafted SCID mice. When CEM cells were removed from spleens, livers and tumors of mice implanted with 2F5 neo-organs, very low reverse transcriptase activity was consistently observed, in comparison with cultures obtained from HIV-infected control SCID.

Example 9: *In vivo* decrease of viral load induced by sCD4-IgG and 2F5mAb and sCD4-IgG

***A) In vivo* decrease of plasma viral load induced by sCD4-IgG**

A separate experiment to compare 2F5mAb with sCD4-IgG showed that both compounds have comparable inhibitory effects on the plasma viral load of HIV Lai- or HIV MN-infected SCID-CEM mice as illustrated in Table 3. As a stock of Lai virus different from that in the previous experiment (Table 2) was used, the mean plasma viral load was higher. However it was significantly lower in all groups of implanted mice than in controls.

Table 3

Mice	HIV-1-RNA Copies/ml at : Day 0	Day 4	Day 10	Day 15
Controls				
SCID 01 + Lai	<1200	68 x 10^6	100 x 10^6	110 x 10^6
SCID 02 + Lai	<1100	96 x 10^6	98 x 10^6	160 x 10^6
SCID 03 + Lai	<870	100 x 10^6	120 x 10^6	100 x 10^6
SCID 04 + Lai	< 1400	130 x 10^6	95 x 10^6	230 x 10^6
SCID 05 + Lai	<890	80 x 10^6	110 x 10^6	150 x 10^6
SCID 06 (- Lai)	<2000	<1100	<770	<890
SCID 07 (- Lai)	<1100	<760	<540	<540
SCID 08 (- Lai)	<640	<1100	< 600	<1100
2F5 neo-organs				
SCID 09 + Lai	<1200	35 x 10^5	15 x 10^5	7,2 x 10^5
SCID 10 + Lai	<740	<1100	25 x 10^5	3,5 x 10^5
SCID 11 + Lai	<810	36 x 10^5	8,1 x 10^5	4,0 x 10^5
SCID 12 + Lai	<970	<2000	11 x 10^5	1,2 x 10^5
SCID 13 + Lai	<860	49 x 10^5	19 x 10^5	1,8 x 10^5
SCID 14 + Lai	<790	15 x 10^5	15 x 10^5	14 x 10^5
sCD4-IgG neo-organs				
SCID 15 + Lai	<750	27 x 10^5	16 x 10^5	6,4 x 10^5
SCID 16 + Lai	<900	38 x 10^5	23 x 10^5	8,7 x 10^5
SCID 17 + Lai	<1100	43 x 10^5	23 x 10^5	2,2 x 10^5
SCID 18 + Lai	<2600	57 x 10^5	15 x 10^5	2,7 x 10^5
SCID 19 + Lai	<800	64 x 10^5	2,2 x 10^5	9,1 x 10^5
SCID 20 + lai	<860	0,25 x 10^5	16 x 10^5	2,6 x 10^5
2F5 + sCD4-IgG neo-organs	<			
SCID 21 + Lai	<1100	28 x 10^5	8,0 x 10^5	6,7 x 10^5
SCID 22 + Lai	<1600	21 x 10^5	5,1 x 10^5	11 x 10^5
SCID 23 + Lai	<500	27 x 10^5	5,0 x 10^5	2,4 x 10^5
SCID 24 + Lai	<780	40 x 10^5	4,8 x 10^5	5,1 x 10^5
SCID 25 + Lai	<570	16 x 10^5	8, 3 x 10^5	7,2 x 10^5
SCID 26 + Lai	<1400	27 x 10^5	3,2 x 10^5	1,1 x 10^5
SCID 27 + Lai	<1600	36 x 10^5	7,7 x 10^5	1,7 x 10^5

Statistical analysis determined using a paired t-test

2F5 neo-organs versus controls P<0.01.

sCD4-IgG neo-organs versus controls P<0.01.

2F5 + sCD4-IgG neo-organs versus controls P<0.01.

sCD4-IgG neo-organs versus 2F5 neo-organs not statistically significant

***B) In vivo* decrease of cellular viral load induced by sCD4-IgG**

The effect of sCD4-IgG and 2F5 were therefore of the same order of magnitude.

At the time of death, the number of HIV-1 proviral DNA copies was low in the spleen cells of mice implanted with neo-organs secreting sCD4-IgG compared with controls (Table 4).

Table 4

Summary of HIV-1 proviral DNA detection, by quantitative-competitive polymerase chain reaction, in spleen cells of control, 2F5 or sCD4-IgG or 2F5 + sCD4-IgG neo-organ-grafted, SCID mice, infected with HIV-1 (Lai).

Competitor copies	$3x10^5$	$3x10^4$	$3x10^3$	$3x10^2$
Controls				
SCID 01 + Lai	X*			
SCID 02 + Lai	X			
SCID 03 + Lai	X			
2F5 neo-organs				
SCID 09 + Lai			X	
SCID 10 + Lai			X	
SCID 11 + Lai				X
SCID 12 + Lai		X		
SCID 13 + Lai		X		
sCD4-IgG				
SCID 15 + Lai			X	
SCID 16 + Lai		X		
SCID 17 + Lai		X		
SCID 18 + lai	X			
SCID 19 + Lai	X			
2F5 + sCD4-IgG				
SCID 21 + Lai			X	
SCID 22 + Lai		X		
SCID 23 + Lai			X	
SCID 24 + Lai			X	
SCID 25 + Lai			X	
SCID 26 + Lai			X	

*:Crosses determine the equimolarity of the copy number of the HIV-1 *gag* fragment between the target DNA and the competitor DNA.

The Table shows that the efficacy of [2F5 plus sCD4-IgG] is better by a ten factor than any of 2F5 and sCD4-IgG, and that is efficacy is almost the same for all tested mice.

***C) In vivo* decrease of plasma viral load induced by both 2F5 and sCD4-IgG**

When both molecules were produced together *in vivo*, again an important anti-HIV activity was demonstrable (Table 4).

***D) In vivo* decrease of cellular viral load induced by both 2F5 and sCD4-IgG**

At the time of death, the number of HIV-1 proviral DNA copies was low in the spleen cells of mice implanted with neo-organs secreting 2F5 plus sCD4-IgG compared with controls (Table 5). In this group, a more homogeneous and potent effect was observed compared to neo-organ secreting only one of 2F5 and sCD4-IgG.

Example 10: Cooperation between secreted 2F5 and sCD4IgG

The production *in vivo* of 2F5 and sCD4 in SCID mice of Example 7B, decreases the viral load from 1,5 logarithm on day 4 to 2 logarithm on day 10, as shown on Figure 6.

The effects of 2F5 and sCD4-IgG on the plasmatic viral load *in vivo* in SCID-Hu mice is summarized in Table 5 below.

Table 5

Mice	HIV-1-RNA copies/ml :		
	Day 0	Day 4	Day 10
Controls HIV^+ (5)	1200	$9.6x10^7$	$1.0x10^8$
2F5 + HIV-1 (6)	880	$2.5x10^6$	$1.3x10^6$
SCD4-IgG+ HIV-1 (6)	880	$4.5\ x10^6$	$1.6\ x10^6$
2F5 + sCD4-IgG+ HIV-1 (6)	1100	$2.7\ x10^6$	$0.5\ x10^6$

The cooperation between 2F5 and sCD4-IgG thus secreted allows to reach about ten days after the HIV-1 injection a response three times higher than the one with either 2F5 or sCD4-IgG separately.

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